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Quantitative Analysis of the High Temperature-induced Glycolytic Flux Increase in *Saccharomyces cerevisiae* Reveals Dominant Metabolic Regulation^{*[5]}

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A major challenge in systems biology lies in the integration of processes occurring at different levels, such as transcription, translation, and metabolism, to understand the functioning of a living cell in its environment. We studied the high temperature-induced glycolytic flux increase in *Saccharomyces cerevisiae* and investigated the regulatory mechanisms underlying this increase. We used glucose-limited chemostat cultures to separate regulatory effects of temperature from effects on growth rate. Growth at increased temperature (38 °C versus 30 °C) resulted in a strongly increased glycolytic flux, accompanied by a switch from respiration to a partially fermentative metabolism. We observed an increased flux through all enzymes, ranging from 5- to 10-fold. We quantified the contributions of direct temperature effects on enzyme activities, the gene expression cascade and shifts in the metabolic network, to the increased flux through each enzyme. To do this we adapted flux regulation analysis. We show that the direct effect of temperature on enzyme kinetics can be included as a separate term. Together with hierarchical regulation and metabolic regulation, this term explains the total flux change between two steady states. Surprisingly, the effect of the cultivation temperature on enzyme catalytic capacity, both directly through the Arrhenius effect and indirectly through adapted gene expression, is only a moderate contribution to the increased glycolytic flux for most enzymes. The changes in flux are therefore largely caused by changes in the interaction of the enzymes with substrates, products, and effectors.

Microorganisms encounter environmental changes, which they have to withstand and adapt to in order to survive. Changes in ambient temperature are common to almost every ecological niche. Temperature influences the structural and functional properties of cellular components, both physically

and chemically. Physically, temperature affects membrane fluidity (1, 2) and diffusion rates, as well as protein folding and stability (3). Chemically, temperature directly affects reaction rates in the cell. This study focuses on the adaptation of cells to temperatures higher than that optimal for growth.

Microbes adapt to high temperature by altering their cellular make-up such as lipid composition, membrane fluidity, and the induction of large numbers of heat shock genes (3–11), which have a wide variety of functions. Many encode protein chaperones involved in protein (un)folding (12, 13) or degradation of damaged proteins (14). Others are involved in the synthesis of the thermoprotecting disaccharide trehalose, which is known to be involved in stabilization of membranes and proteins (15, 16) as well as in storage of free energy (17). Many of these adaptive responses put a significant additional energy burden on the cells (18).

There still is little clarity on the actual mechanisms by which cells maintain a balance between the energy needs for adaptive responses to stress survival and those for processes indispensable for growth. To shed more light on this, we quantitatively analyzed the behavior of yeast glycolysis upon a temperature challenge. This well studied catabolic route is central to the free-energy metabolism of the cell, although it is itself also subject to the effects of temperature fluctuations. First, temperature itself has a drastic effect on the catalytic properties of enzymes. The temperature dependence of catalytic rates is partially described by the Arrhenius equation (19). According to this equation, an increase in temperature or decrease in activation energy will result in an increase in activity. Although this relationship may be valid, it represents an oversimplification because it does not take into account the temperature-dependent effects of allosteric factors, nor does it include temperature-dependent degradation. Second, changes in the concentration and/or catalytic capacity of enzymes can change the flux. Such hierarchical regulation could be effected at multiple levels as follows: transcription, mRNA degradation, protein synthesis or degradation, and post-translational modification. Finally, an altered flux could be regulated metabolically. An altered metabolite environment for any enzyme, such as increased substrate, decreased product concentrations, or changed effector concentrations, can drive an increase in *in vivo* reaction rates (20).

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^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table 1.

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Here we studied the quantitative effect of increased temperature on the carbon and energy fluxes of *Saccharomyces cerevisiae* both at the anabolic and catabolic level. Our data were subsequently subjected to regulation analysis (21). To this aim, we investigated whether the effect of temperature on enzyme rates could be included as a separate term. For most enzymes, this was the case. The analysis showed that the increase in glycolytic flux observed at higher ambient temperature is primarily regulated at the metabolic level, whereas contribution of hierarchical regulation and temperature effects is minor.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—*S. cerevisiae* strain CEN.PK113-7D (*MATa MAL2-8^c SUC2*) was cultivated in 500-ml batch fermentors with water jackets (built in-house) for temperature control. Stirring rate was set to 600 rpm and an aeration rate of a fermentor volume of air per min. The culture was kept at pH 5.0 by automatic titration with 0.1 M KOH using an Applikon ADI 1030 Controller (Applikon, Schiedam, The Netherlands). The medium used for cultivation was based on previously described mineral medium (22), supplemented with 20 g liter⁻¹ glucose. Alternatively, strain CEN.PK113-7D was grown in aerobic, carbon-limited 2-liter chemostats (Applikon, Schiedam, The Netherlands) with a working volume of 1 liter at a dilution rate of 0.1 h⁻¹. Precultures were grown overnight in mineral medium with 20 g liter⁻¹ glucose in shake flasks at 30 °C and 200 rpm. The medium used for chemostat cultivation was the same mineral medium as used in batch fermentors, however here containing 7.5 g liter⁻¹ glucose. The stirrer speed was set to 800 rpm, whereas the pH was set to pH 5.0 and kept constant by automatic titration with 1 M KOH. The temperature of the chemostat was controlled with a heat jacket and a temperature probe. Stirring rate, pH, and temperature were kept constant using an Applikon ADI 1010 Biocontroller (Applikon, Schiedam, the Netherlands). The chemostat was aerated by flushing air at 30 liters h⁻¹ through the culture. Steady states were verified by off gas analysis for oxygen and carbon dioxide and by dry weight measurements.

Biomass Dry Weight Measurements—The dry weight concentration was determined in triplicate by filtering 10.0 ml of broth on pre-washed and pre-weighed cellulose acetate membrane filters (pore size 0.45 μm, Schleicher & Schuell). Each filter was washed with 10 ml of demineralized water and dried in a 450-watt microwave (Whirlpool Promicro 825, Sweden) for 15 min. Filters were cooled in a desiccator and weighed on an electronic analytical balance (Mettler-Toledo AB104, Columbus, OH).

Off Gas Analysis—The oxygen and carbon dioxide levels in the exhaust gas of the fermentors were monitored on-line using an oxygen analyzer (Servomex Ltd. Paramagnetic O₂ transducer) and a carbon dioxide analyzer (infrared Servomex Xentra 4100 gas purity analyzer).

Analysis of Metabolites—To analyze glucose, ethanol, glycerol, succinate, acetate, and trehalose, 1.0 ml of broth was quickly quenched in 100 μl of 35% perchloric acid. Samples were subsequently neutralized with 55 μl of 7 M KOH. Culture samples and media samples were analyzed by high pressure liquid chromatography on a Phenomenex Rezex ROA-Organic

Acid H⁺ column using 7.2 mM H₂SO₄ as mobile phase. Glycogen was measured according to Parrou and Francois (23). To analyze residual glucose, 5.0 ml of broth was quickly (within seconds) filtered through 0.2-μm pore size filter (Acrodisc Syringe Filter, Pall Life Science, Ann Arbor, MI). Glucose analysis was performed spectrophotometrically (Pharmacia LKB Novaspec II, Freiburg, Germany) using a Sigma glucose assay kit (catalog number GAGO20-1KT, St. Louis, MO) according to the manufacturer's instructions.

Enzyme Activity Measurements—Enzyme extracts were prepared at 0 °C by adding glass beads followed by thorough sonication according to van Hoek *et al.* (24). The extraction procedure was modified to more closely resemble *in vivo* conditions by performing it in a buffer containing 100 mM K₂SO₄, 10 mM KH₂PO₄ at a pH of 7.0. Enzyme activity assays were carried out according to the protocol described by Rossell *et al.* (25), which uses similar conditions for the determination of all enzyme activities. Assays were performed at both 30 and 38 °C. Protein determination of cell-free extracts was performed using the BCA protein assay method according to the manufacturer's specifications (Pierce).

Glucose Transport Activity Measurements—Zero-trans influx of ¹⁴C-labeled glucose was measured in a 5-s uptake assay described by Walsh *et al.* (26). The assay was carried out in growth medium at assay temperatures of 30 and 38 °C. The data were fit to Michaelis-Menten kinetics with one or two components with SigmaPlot version 7.0 (Systat Software Inc., San Jose, CA).

Distribution of Fluxes—Intracellular metabolic fluxes were determined through metabolic flux balancing using a stoichiometric model according to Daran-Lapujade *et al.* (27).

RNA Isolation—RNA was isolated from the cell using the hot phenol method (28). The amount of RNA was measured with the Novostar (BMG Labtech), and similar amounts of RNA were used in the cDNA reaction. Genomic DNA was removed by a DNase I treatment (1 unit; Ambion or Roche Applied Science). cDNA was synthesized using Moloney murine leukemia virus H⁻ (Bioke, The Netherlands) and hexanucleotides (Bioke, The Netherlands).

qPCR² Analysis—Oligonucleotide primers were designed to amplify an 80–120-bp amplicon. Protein-disulfide isomerase 1 (PDI1) was chosen as an internal standard. Primers were designed using Primer Express software 1.0 (PE Applied Biosystems, Foster City, CA). PCRs (10 μl) were set up and run as described by the manufacturer. Briefly, the reactions contained 5 μl of SYBR Green PCR core kit (Bioke, The Netherlands), 3 pmol of each primer (Biolegio or Isogen), and 0.1 μl of cDNA template (equivalent to 1 ng of RNA). Amplification, data acquisition, and data analysis were carried out in the 7900HT fast real time PCR system (Applied Biosystems) (once at 2 min, 50 °C; 10 min, 95 °C; and 40 cycles at 95 °C, 15 s; 60 °C, 1 min). The calculated cycle threshold values (*Ct*) were exported to

² The abbreviations used are: qPCR, quantitative PCR; HXK, hexokinase; PGI, glucose-6-phosphate isomerase; PFK, phosphofructokinase; TPI, triose-phosphate isomerase; TDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, 3-phosphoglycerate kinase; ADH, alcohol dehydrogenase; LC-ESI-MS/MS, liquid chromatography/tandem mass spectrometry.

Temperature Regulation of Yeast Glycolysis

Microsoft Excel for analysis using the *DDCt* method (29) and normalized to PDI1 and then to the 30 °C sample. Dissociation curves (dissociation curves 1.0 f. software, PE Applied Biosystems, Foster City, CA) of PCR products were run to verify amplification of the correct product.

Analysis of Glycolytic Metabolites—Samples from at least two independent chemostat cultures were taken using a rapid sampling setup (30) and quenched using the cold methanol quenching method as described by Mashego *et al.* (31). Glycolytic metabolites were analyzed by LC-ESI-MS/MS according to van Dam *et al.* (32), and adenosine nucleotides were analyzed by LC-ESI-MS/MS according to Wu *et al.* (33). All LC-ESI-MS/MS analyses were done in duplicate, and intracellular metabolite quantification was performed by applying the isotope dilution method (34).

Immunoblotting—Cell-free extracts were prepared using a Fast Prep bead beater (Bio101 Savant, Inc.). To break the cells, 6 cycles of 20 s at speed 4 was sufficient. Between each cycle samples were cooled on ice for 60 s. Breakage of the cells was checked by microscopy. A protease inhibitor mixture (Sigma) was used to inhibit protease activity. The extracts were centrifuged 5 min at 10,000 rpm to remove insoluble particles. The protein determination of the extracts was performed using the

BCA protein assay method according to the manufacturer's protocol (Pierce). Samples were assayed on a Phast System (Amersham Biosciences) using 12.5% SDS-polyacrylamide gels. Proteins were transferred to a nitrocellulose filter by diffusion for 2 h, and the gel was stained afterward with Coomassie Brilliant Blue. Subsequently, the filter was blocked in phosphate-buffered saline containing 0.1% Tween and 1% Protifar (Nutricia, The Netherlands) and decorated with polyclonal antibodies against PGI, PGK, and TPI (Nordic Immunology, Tilburg, The Netherlands). GARPO secondary antibodies (Sigma), ECL detection, and densitometric scanning of the resulting film were used to quantify the bands with cross-reactive material.

RESULTS

Increased Temperature Leads to an Increase in Glycolytic Flux—To study the effect of temperature on growth rate, *S. cerevisiae* strain CEN.PK113-7D was grown in aerated, pH- and temperature-controlled glucose excess batch fermentors at temperatures in the range of 27–41 °C (Fig. 1). First, in the lower temperature range, an increase of relative growth rate with temperature was observed, with a maximum growth rate at 33 °C. Increasing the temperature further to 37 °C had only a moderate effect, although further increase of temperature led to a decreased growth rate.

To separate the effect of temperature on glycolytic flux from the effect of changes in growth rate on metabolism, we used carbon-limited chemostats, in which we set the dilution rate to control culture growth rate. We used a dilution rate of 0.1 h⁻¹ to analyze the effect of temperature on glycolysis. Cultures were assumed to be in steady state when the O₂ and CO₂ concentrations in the off-gas were constant for more than 1 day (2.4 residence times). From these cultures the overall steady state carbon fluxes were analyzed. Fig. 2 shows the temperature dependence of fluxes and biomass dry weight. The concentrations of residual glucose, succinate, acetate, and pyruvate were below the detection limit in all cultivations. The specific oxygen consumption rate was constant (~3 mmol g dry weight⁻¹ h⁻¹) at all temperatures, although there was no detectable flux toward ethanol in the range of 30–37 °C. Surprisingly, the latter steeply increased above 37 °C. This implies that in the range of 30–37 °C, the consumed glucose is completely respired, whereas above 37 °C cells grow with a respiro-fermentative metabolism, which yields less energy. We used the fluxes of CO₂, ethanol, glucose, and glycerol to calculate the fluxes through the individual enzymes of glycolysis at 30 and 38 °C (Fig. 3). These conditions were chosen because they have the same μ_{\max} when grown in batch fermentors, and therefore the cells grow at the same rate relative to μ_{\max} (Fig. 1). We observed a 5–10-fold increased flux through all glycolytic enzymes in cultures at 38 °C when compared with 30 °C, and we asked how this increase was accomplished.

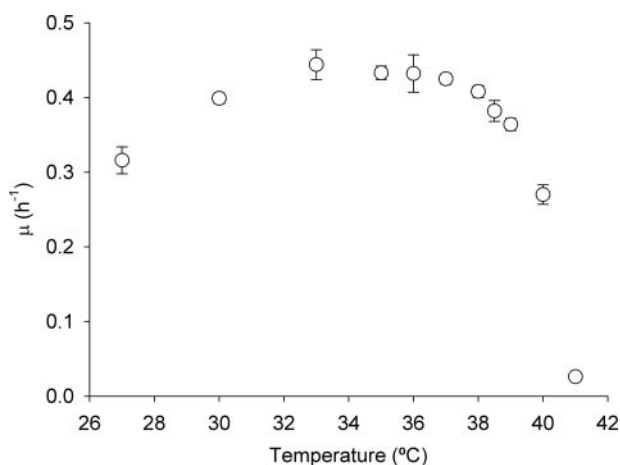


FIGURE 1. **Effect of temperature on the maximal growth rate.** Specific growth rate \pm S.D. of at least three independent batch fermentors was measured at various temperatures in the range of 27–41 °C.

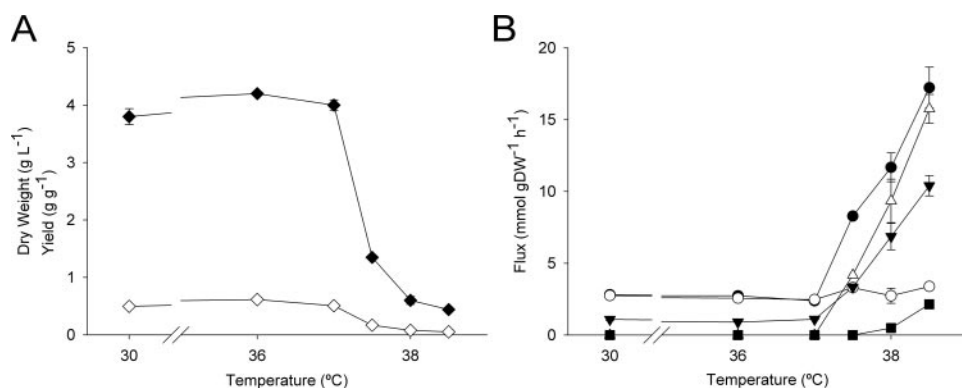


FIGURE 2. **Temperature effect on physiological characteristics of glucose limited aerobic chemostat cultures grown at various temperatures in the range of 30–39 °C.** A, effect of temperature on dry weight (solid diamonds) and yield (open diamonds). B, effect of temperature on glucose flux (solid squares), carbon dioxide flux (solid circles), oxygen flux (open circles), ethanol flux (open triangles), and glycerol flux (solid triangles).

Temperature Regulation of Glycolytic Enzymes—To explain the increased fluxes through all glycolytic enzymes at 38 °C compared with 30 °C, we considered three possible contributions. First, temperature could have a strong direct effect on the

catalytic properties of the enzymes (19), *i.e.* on their V_{\max} . Second, changes in the cellular content of rate-controlling enzymes could cause the flux increases (35). Finally, changes in the metabolite environment of an enzyme, such as substrate and effector concentrations, could underlie the changes in flux (36). In addition, any combination of the above mentioned types of regulation could occur (25).

To assess the direct effect of assay temperature on catalytic properties of glycolytic enzymes, we determined the temperature dependence of the V_{\max} *in vitro*. The V_{\max} was measured at 30 and 38 °C in cell extracts from chemostat cultivations grown at 30 or 38 °C. In this way we were able to isolate the direct effect of temperature on V_{\max} from that of the adaptive response of the cell to the temperature increase. The latter might also cause changes in the catalytic rate of the enzyme *in vivo*.

The direct effect of temperature on enzyme catalytic rates in cell extracts isolated from cultivations at 30 °C ranged from 0.6-fold, for phosphofructokinase (PFK), to 1.7-fold, for triosephosphate isomerase (TPI) (Table 1). The rates of the other enzymes were not significantly affected by the increase in temperature. Similarly, in cell extracts isolated from cultivations at 38 °C, the direct effect of temperature ranged from 0.5- to 1.9-fold (Table 1). In these cell extracts the hexose transporters, hexokinase (HXX), fructose-1,6-bisphosphate aldolase, and PGK did not show a significant change in V_{\max} when assayed at both temperatures. To show that we were not observing an artifact introduced by enzyme inactivation during the assay, we tested the activity of HXX, which belongs to the most thermostable enzymes (37), after incubation of cell-free extracts from steady states at both cultivation temperatures at 38 °C for 30 min. No significant decrease in enzyme capacity was observed (data not shown). The direct effect of assay temperature on activity of cell extracts from 38 °C cultivations was in most cases not significantly different from that on the activity of cell extracts from 30 °C cultivations (ratio 38/30 °C). Notable exceptions were phosphoglucose isomerase (PGI), PFK, and glyceraldehyde-3-phosphate dehydrogenase (TDH) (Table 2),

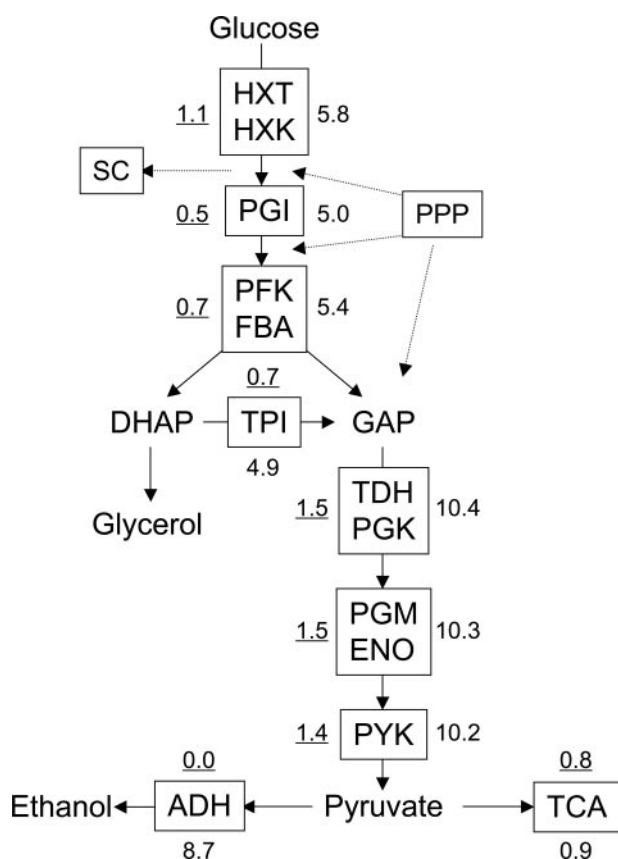


FIGURE 3. Stoichiometry of the glycolytic, fermentative, and tricarboxylic acid cycle (TCA) pathway. We calculated the *in vivo* fluxes through glycolysis. In this simplified scheme enzymes with the same flux are boxed together. The numbers next to the boxed enzymes are the calculated *in vivo* fluxes through the enzymes of 30 °C (underlined) and 38 °C (normal numbers) cultivations. Many enzyme abbreviations are defined in the abbreviation footnote. The following abbreviations are used: SC, storage carbohydrates; PPP, pentose-phosphate pathway; FBA, fructose-1,6-bisphosphate aldolase; PYK, pyruvate kinase.

TABLE 1

***In vitro* enzyme activity determination of *S. cerevisiae* cell-free extracts, grown in glucose-limited aerobic chemostats at 30 and 38 °C**

Values represent the mean \pm S.D. of four independent chemostats. The assay temperature effect is expressed as the ratio of enzyme activities measured at 38 °C over those measured at 30 °C for one culture temperature. The culture temperature effect is expressed as the ratio of enzyme activities measured at a single assay temperature for cultures grown at 38 °C over those of cultures grown at 30 °C. The following abbreviations are used: HXT, hexose transporter; FBA, fructose-1,6-bisphosphate aldolase; GPM, phosphoglycerate mutase; PYK, pyruvate kinase.

Enzyme	Culture temperature 30 °C, assay temperature 30 °C	Culture temperature 30 °C, assay temperature 38 °C	Culture temperature 38 °C, assay temperature 30 °C	Culture temperature 38 °C, assay temperature 38 °C	Assay temperature effect		Culture temperature effect	
	A	B	C	D	B/A	D/C	C/A	D/B
<i>mmol·min⁻¹·g protein⁻¹</i>								
HXT	0.3 \pm 0.0	0.3 \pm 0.0	0.2 \pm 0.1	0.2 \pm 0.0	1.1	1.0	0.8	0.7
HXX	0.4 \pm 0.1	0.5 \pm 0.1	0.6 \pm 0.2	0.8 \pm 0.2	1.3	1.4	1.4	1.5
PGI	1.0 \pm 0.1	1.1 \pm 0.1	1.1 \pm 0.2	1.7 \pm 0.2	1.1	1.5 ^a	1.1	1.6 ^b
PFK	0.3 \pm 0.0	0.2 \pm 0.0	0.4 \pm 0.0	0.5 \pm 0.0	0.6 ^b	1.2 ^b	1.1	2.3 ^b
FBA	0.4 \pm 0.1	0.5 \pm 0.2	0.4 \pm 0.1	0.4 \pm 0.1	1.3	1.2	1.0	0.9
TPI	3.2 \pm 0.5	5.4 \pm 0.3	4.4 \pm 1.1	8.3 \pm 0.3	1.7 ^b	1.9 ^b	1.4	1.5 ^b
TDH	0.6 \pm 0.2	0.5 \pm 0.1	1.4 \pm 0.1	0.7 \pm 0.2	0.8	0.5 ^b	2.2 ^b	1.5 ^a
PGK	3.8 \pm 1.7	3.7 \pm 1.7	11 \pm 0.9	11 \pm 0.9	1.0	1.0	2.8 ^b	2.9 ^b
GPM	2.4 \pm 1.4	2.0 \pm 1.5	4.5 \pm 0.3	4.1 \pm 0.2	0.9	0.9 ^a	1.9 ^a	2.0 ^a
PYK	2.0 \pm 0.9	1.5 \pm 1.1	2.6 \pm 0.1	1.6 \pm 0.2	0.7	0.6 ^b	1.3	1.1
ADH	2.7 \pm 1.9	4.1 \pm 2.0	2.0 \pm 0.3	2.8 \pm 0.2	1.5	1.4 ^b	0.7	0.7

^a *p* value <0.05 was determined from a two-tailed *t*-test assuming equal variance.

^b *p* value <0.01 was determined from a two-tailed *t*-test assuming equal variance.

TABLE 2
Comparison of the effect of assay temperature on 30 and 38 °C cultivations

We compared the ratio of *in vitro* enzyme activity measured at 38 and 30 °C in cell extracts from steady state cultures grown at 30 °C with that measured in cell extracts from 38 °C cultivation. Values represent the fold change \pm S.E. of the activity of one enzyme from one extract measured at two assay temperatures. *p* values indicate the likelihood that the effect of assay temperature on activities of enzymes from 38 °C cultures is the same as that on activities of enzymes from a 30 °C cultivations, analyzed with a two-tailed *t*-test. The following abbreviations are used: HXT, hexose transporter; FBA, fructose-1,6-bisphosphate aldolase; GPM, phosphoglycerate mutase; PYK, pyruvate kinase.

Culture temperature	HXT	HXK	PGI	PFK	FBA	TPI	TDH	PGK	GPM	PYK	ADH
30 °C	1.1 \pm 0.1	1.3 \pm 0.2	1.1 \pm 0.1	0.6 \pm 0.0	1.3 \pm 0.3	1.7 \pm 0.1	0.8 \pm 0.1	1.0 \pm 0.3	0.9 \pm 0.4	0.7 \pm 0.3	1.5 \pm 0.6
38 °C	1.0 \pm 0.2	1.4 \pm 0.4	1.5 \pm 0.2	1.2 \pm 0.1	1.2 \pm 0.2	1.9 \pm 0.2	0.5 \pm 0.1	1.0 \pm 0.1	0.9 \pm 0.0	0.6 \pm 0.0	1.4 \pm 0.1
<i>p</i> value	9.1E-01	4.0E-01	3.0E-04	3.0E-02	7.8E-01	4.4E-01	1.7E-02	2.6E-01	2.6E-01	7.3E-01	4.5E-01

which suggests that their mode of regulation differs significantly at both temperatures.

Because the V_{\max} depends on the amount of enzyme and a rate constant, the effect of increased expression can be determined by comparing the activity of cell-free extracts from two cultivation temperatures measured at one temperature. Only the V_{\max} of TDH and PGK differed significantly in extracts from 38 to 30 °C, when we determined the V_{\max} at 30 °C (Table 1). The same samples measured at 38 °C showed more enzymes that were significantly different in activity (Table 1). However, the activity of HXK, fructose-1,6-bisphosphate aldolase, phosphoglycerate mutase, pyruvate kinase, and alcohol dehydrogenase (ADH) was not significantly different when comparing the activity of extracts from two cultivation temperatures at a 38 °C assay temperature. In conclusion, the temperature-induced 5–10-fold flux increase through glycolysis cannot be explained exclusively by either a direct temperature effect in the catalytic rate or an increased expression of glycolytic enzymes, because these were at most 2.9-fold increased, but generally revealed no significant increase.

Quantitative Contribution of Temperature in Flux Regulation—To quantitatively analyze the contribution of the gene expression cascade, the contribution of increased temperature, and the contribution of metabolic processes to the increase in fluxes through all glycolytic enzymes, we used regulation analysis (21, 38). This analysis makes use of the fact that at steady state, for a linear path, the pathway flux J through an enzyme equals the *in vivo* rate v at which the enzyme catalyzes its reaction.

Enzyme reaction rates are governed by the concentrations and catalytic activities of the enzymes, and their interactions with substrates, products, and effectors. Therefore, they usually have the shape shown in Equation 1,

$$v = f(e) \cdot g(X) \quad (\text{Eq. 1})$$

in which v is the enzyme rate; e is the concentration of enzyme, and X is the vector of substrate, product, and other effector concentrations. Important in this equation is that $f(e)$, which equals the V_{\max} of the enzyme, is independent of X , whereas $g(X)$ is independent of e . Through the Arrhenius equation, temperature affects all elementary rate constants k directly as shown in Equation 2,

$$k(T) = Ae^{-Ea/RT} \quad (\text{Eq. 2})$$

in which A is a constant; Ea is the activation energy of the reaction; R is the gas constant, and T is the absolute temperature. As V_{\max} is the product of an elementary rate constant (k_{cat}) and the enzyme concentration, the function $f(e)$, can be

replaced by $f^*(e) \times q(T)$, which yields Equation 3,

$$v = f^*(e) \cdot q(T) \cdot g(X) \quad (\text{Eq. 3})$$

We consider a comparison of two steady states, at which any enzyme rate v is equal to the flux J . Therefore, we can rewrite Equation 3 as Equation 4,

$$1 = \frac{\Delta \log f^*(e)}{\Delta \log J} + \frac{\Delta \log q(T)}{\Delta \log J} + \frac{\Delta \log g(X)}{\Delta \log J} = \rho_h + \rho_T + \rho_m \quad (\text{Eq. 4})$$

in which ρ_h is the hierarchical regulation coefficient, which expresses how much of the flux regulation is because of changes in enzyme concentration (*i.e.* through gene expression); ρ_T is the contribution of direct regulation of V_{\max} by temperature through the Arrhenius effect; and ρ_m is the metabolic regulation coefficient, which quantifies the contribution of changes in the interaction of the enzyme with the rest of metabolism in relation to the change in flux. The “ Δ ” expresses the difference between two steady states, *e.g.* at different temperatures. Temperature effects on the affinities of the enzymes toward metabolites are also classified in the metabolic term, and must be dissected separately.

We can determine these regulation coefficients from the data collected above. ρ_T is determined from Equation 5,

$$\rho_T = \frac{\Delta \log V_{\max,T}}{\Delta \log J} \quad (\text{Eq. 5})$$

in which the $\Delta \log V_{\max,T}$ is determined by analyzing one sample at two assay temperatures. ρ_h is determined according to the same formula, but now $\Delta \log V_{\max,T}$ is determined by comparing samples from cultures grown at different temperatures but analyzed at one assay temperature. ρ_T can be dissected from ρ_h only if the direct effect of temperature on V_{\max} is independent from that of the gene expression cascade on V_{\max} . The dissection becomes invalid for example if under the two culture conditions cells express different isoenzymes with different temperature dependences. We therefore assessed whether the $\Delta \log V_{\max,T}$ obtained by assaying one sample at two temperatures is identical to that of another sample from a different cultivation temperature (Table 2). In three cases this was not the case (PGI, PFK, and TDH). For these enzymes we determined one combined (ρ_h plus ρ_T) from samples from two cultivation temperatures, each assayed at its cultivation temperature. The metabolic regulation coefficient, ρ_m , was derived from the summation theorem (Equation 4).

TABLE 3

Regulation analysis of local flux changes by temperature increase

The contribution of hierarchical regulation (through the gene expression cascade), temperature regulation (through direct effects of temperature on enzyme activity), and metabolic regulation (through changes in the metabolic environment of the enzyme) were calculated for all glycolytic enzymes. Values represent the mean \pm S.E. of the regulation coefficients determined. The following abbreviations are used: HXT, hexose transporter; FBA, fructose-1,6-bisphosphate aldolase; GPM, phosphoglycerate mutase; PYK, pyruvate kinase.

	P_T	P_h	P_m
HXT	0.0 ± 0.1	-0.1 ± 0.1	1.1
HXX	0.2 ± 0.0	0.2 ± 0.1	0.6
PGI		0.2 ± 0.0	0.8
PFK		0.2 ± 0.0	0.8
FBA	0.1 ± 0.0	0.0 ± 0.0	0.9
TPI	0.3 ± 0.0	0.2 ± 0.0	0.5
TDH		0.1 ± 0.1	0.9
PGK	0.0 ± 0.0	0.5 ± 0.1	0.5
GPM	-0.1 ± 0.0	0.3 ± 0.1	0.8
PYK	-0.2 ± 0.0	0.1 ± 0.1	1.1
ADH ^a	0.1 ± 0.0	0.0 ± 0.1	0.9

^a The flux for ethanol in 30 °C steady state cultures can not be determined since the concentration of ethanol is below the detection limit. Assuming a small production of 0.4 mM ethanol (detection limit), we calculated a flux through ADH of 0.01 mmol g⁻¹ h⁻¹ and used this flux for regulation analysis. Increasing or decreasing this flux 10-fold did not significantly affect regulation analysis of this enzyme.

We used the adapted regulation summation theorem to analyze in what way yeast cells bring about a flux change in response to an increase in culture temperature. The results of the regulation analysis are summarized in Table 3. For ADH no local flux increase could be determined because of the absence of ethanol fermentation in cultivations at 30 °C, and therefore regulation analysis is not applicable. However, we could calculate the flux through this enzyme assuming an ethanol concentration of around the detection limit. When we performed regulation analysis with this flux, as well as one of 10 times higher or lower, we found that the regulation of flux through ADH was not drastically affected, as in all cases it was almost exclusively regulated metabolically (Table 2). In fact, substantial contributions of hierarchical regulation to flux are found only for PGK and phosphoglycerate mutase, although in the case of HXX and TPI hierarchical regulation and temperature regulation of enzyme activity together contribute significantly to the flux increase. However, in general, rate increases caused by temperature directly or caused by increased enzyme expression are only minor contributions to the flux increase. This implies that the increase in flux through glycolytic enzymes in response to a temperature increase from 30 to 38 °C is regulated by changes in metabolic environment.

mRNA and Protein Levels of Glycolytic Enzyme Encoding Genes Are Largely Unchanged—To confirm the small contribution of the gene expression cascade in relation to the local temperature-induced flux increase, we measured glycolytic pathway mRNA levels in extracts from our chemostat cultivations grown at 38 and 30 °C, using qPCR. Of the 30 glycolytic genes measured, 11 were significantly altered in expression at 38 °C compared with 30 °C (supplemental Table 1). The most striking difference in expression was found for *ADH2*. In cultivations grown at 38 °C, the *ADH2* mRNA was down-regulated 277-fold. Strikingly, this strong down-regulation in gene expression resulted in only a small decrease in enzyme activity (Table 1). In several cases the relative expression of different isoenzymes contributing to one activity was altered. For instance, *HXX1* was up-regulated, while *GLK1* was reduced in expression.

TABLE 4

Steady state intracellular metabolite levels of chemostat cultures at 30 and 38 °C

Values represent the mean (in $\mu\text{mol g dry weight}^{-1}$) \pm S.D. of data of six samples from at least two independent chemostats. Values for residual glucose represent the mean (in μM) \pm S.D. of data from four independent steady-state chemostat cultivations. The following abbreviations are used: Glc out, residual glucose; Fru-6-P, fructose 6-phosphate; Glc-6-P, glucose 6-phosphate; Glc-3-P, glucose 3-phosphate; 2PG+3PG, 2-phosphoglycerate and 3-phosphoglycerate; T6P, trehalose 6-phosphate; F1,6bP, fructose 1,6-bisphosphate; F2,6bP, fructose 2,6-bisphosphate.

	Culture temperature		FC, 38/30 °C
	30 °C	38 °C	
Nucleotides			
AMP	0.6 ± 0.2	0.6 ± 0.0	1.0
ADP	1.3 ± 0.3	1.7 ± 0.1	1.3 ^a
ATP	7.0 ± 1.4	5.3 ± 0.4	-1.3 ^a
Glycolysis			
Glc out	18 ± 9.4	194 ± 100	11 ^a
Glc-6-P	2.9 ± 0.8	3.9 ± 0.5	1.3 ^a
Fru-6-P	0.6 ± 0.2	0.4 ± 0.1	-1.5 ^a
Fru-1,6bP	0.8 ± 0.4	7.5 ± 2.5	9.5 ^a
Glc-3-P	0.1 ± 0.0	0.9 ± 0.1	16 ^a
2PG+3PG	1.4 ± 0.4	0.3 ± 0.1	-5.5 ^a
Phosphoenolpyruvate	1.2 ± 0.4	0.1 ± 0.0	-15 ^a
Pyruvate	0.3 ± 0.1	6.7 ± 2.5	19 ^a
Glycolytic branches			
F2,6bP	0.002 ± 0.003	0.013 ± 0.007	8.6 ^a
T6P	0.3 ± 0.1	0.1 ± 0.0	-4.0 ^a

^a Significance of the change as determined with a two-tailed *t* test, *p* < 0.01.

The correlation between mRNA and corresponding protein level was studied several times and showed that mRNA abundance alone is an insufficient indication of corresponding protein level (39, 40). We therefore compared the enzyme levels of 30 °C cells with 38 °C cells by using immunoblotting. Western blot analysis for PGI, TPI, and PGK revealed no significant change in PGI and TPI protein level comparing 30 and 38 °C steady state cultures. The amount of PGK was increased (data not shown), qualitatively corroborating the strongly increased activity for this enzyme (see Table 1) and the contribution of this up-regulation to the *in vivo* flux increase through this enzyme.

Intracellular Glycolytic Metabolite Concentrations Are Affected by Temperature Increase—Regulation analysis revealed that, in general, the local flux increase caused by a temperature up-shift is caused by changes in the interactions of the enzymes with their metabolic environment. Therefore, we analyzed the concentrations of glycolytic metabolites. In contrast to the limited changes in enzyme activities and mRNA concentrations, the concentrations of all measured glycolytic intermediates were significantly changed (Table 4). The concentrations of extracellular glucose (Glc out), glucose 6-phosphate, fructose 1,6-bisphosphate, glycerol 3-phosphate, and pyruvate were significantly increased ranging from 1.3- to 19-fold in cells cultivated at 38 °C compared with cells grown at 30 °C (Table 4). The concentration of intermediates fructose 6-phosphate, 2-phosphoglycerate, and 3-phosphoglycerate and phosphoenolpyruvate was significantly decreased (1.5–15-fold). The concentration of glycerol 3-phosphate increased 16-fold, which coincides with the production of glycerol observed in cultures grown at 38 °C. The concentration of trehalose 6-phosphate decreased significantly (4.0-fold). Trehalose 6-phosphate is involved in the biosynthesis of trehalose, and it is a known inhibitor of the hexokinase activity (41). The decrease in trehalose 6-phosphate may thus contribute to the increased flux through hexokinase.

The concentration of the known activator of PFK, fructose 2,6-bisphosphate, is increased 8.6-fold in 38 °C grown cells. This increase may contribute in keeping PFK converting fructose 6-phosphate into fructose 1,6-bisphosphate, even when the concentration of the latter is high. In turn, a higher level of fructose 1,6-bisphosphate, which is a potent activator of pyruvate kinase (42–44), could help maintain a high flux through pyruvate kinase at 38 °C, even when the concentration of its substrate, phosphoenolpyruvate, is much lower. As for the pyruvate branch point, which is the intersection of glycolysis with the tricarboxylic acid cycle and C₂ metabolism, a much higher intracellular concentration of pyruvate at 38 °C is in agreement with the occurrence of alcoholic fermentation, because high intracellular concentrations of pyruvate are thought to favor its dissimilation via pyruvate decarboxylase (45). Finally the levels of adenosine nucleotides, which reflect the balance between ATP supply and demand, can also affect the activities of glycolytic enzymes. Comparing the AXP concentrations of steady state cultures at 38 °C with cultures grown at 30 °C showed no change in the concentration of AMP. The concentration of ATP was decreased 1.3-fold at 38 °C, and the concentration of ADP increased 1.3-fold. As it is well known that the intracellular ATP concentration is inversely correlated to the glycolytic flux (46), this may also contribute to the observed increase of the latter at 38 °C.

DISCUSSION

Temperature affects all processes in living organisms. Focusing on a defined pathway, we studied how *S. cerevisiae* regulates its systems properties when challenged with an increase in ambient temperature. Elaborate studies have studied the regulation of fluxes in response to nutrient starvation (25) and regulation of fluxes in response to gene deletions (47) using regulation analysis. Different modes of regulation were observed. This study is the first example in which regulation analysis was applied to determine the quantitative contribution of the direct temperature effects on enzyme kinetics, the effects of changes in gene expression, and an altered metabolic make-up of the cell to the increased flux through the glycolytic enzymes. We adapted regulation analysis and showed that a ρ_T can be included as a separate term for most enzymes. Next, we used the adapted regulation analysis to determine the mechanisms by which the glycolytic flux increase is regulated upon a temperature up-shift. We found that neither a direct effect of temperature on enzyme catalytic rates nor an adaptive response affecting enzyme capacity through the gene expression cascade contributed much to the flux increase through most enzymes. Rather, around 85% of the flux increase must be brought about by changes in the metabolic environment of each enzyme.

Possibly the most remarkable aspect of the effect of temperature on glycolytic flux is the fact that yeast shifts from a respiratory metabolism to a respiro-fermentative metabolism, where the ATP yield on the limiting substrate, glucose, is lower. Yet cells need more energy because of higher maintenance requirements at higher temperatures. We postulated three hypotheses to explain this shift. First, the cells at 30 °C might already have reached a maximal respiratory capacity, which cannot be increased any further. However, preliminary experi-

ments in which we added sorbic acid, a known uncoupler (see e.g. Ref. 48), to 30 °C steady state cultures show that oxygen consumption can be strongly increased,³ which is inconsistent with a hypothesis in which maximal mitochondrial capacity has already been reached. Second, mitochondria or mitochondrial metabolism might be much more sensitive to high temperatures, for example because of the membrane composition of the mitochondrial membrane (49) or because of a temperature sensitivity of mitochondrial protein import (50). An increased incidence of petite mutants has also been reported (51), but this was not the case in our experiments.³ Third, mitochondrial metabolism at 38 °C might become harmful to the cell for some unknown reason, and therefore the flux through the tricarboxylic acid cycle is inhibited at higher temperatures.

To quantify the contributions of various modes of enzyme flux regulation, we used regulation analysis. We first determined that a separate regulatory term ρ_T could be introduced by assaying glycolytic enzyme activity at 30 and 38 °C in cell extracts from steady state chemostat cultures at both temperatures. Our assays indicated that for most enzymes the direct effect of temperature on enzyme catalytic rates was independent from culture temperature, allowing us to introduce a ρ_T . Remarkably, the direct effect of high temperature on the activity of almost all glycolytic enzymes was moderate (Table 2). The temperature dependence of an enzymatic rate is often expressed as the Q_{10} , which is a measure of the change of the reaction rate of a chemical or biological reaction as a consequence of increasing the temperature by 10 °C. As a rule of thumb, this Q_{10} is thought to be ~2, but in reality it depends on the activation energy of the reaction of interest. We found that, except for TPI, the Q_{10} for all glycolytic enzymes was well below 2. This is in marked contrast to data from a recent study on cultivations at a low temperature where the temperature dependence of enzyme activity was shown to be very strong (52). The experiments by Tai *et al.* (52) show that for more enzymes the difference in rates between 12 and 30 °C is much closer to the expected Q_{10} of 2. Together, these data suggest that enzyme rate increases are somehow buffered at temperatures above the optimal growth temperature of the organism, possibly to prevent an overshoot of metabolism at high temperatures.

Besides a very low impact of temperature on enzyme rate directly, we also found that their capacity was rarely up-regulated through increased expression. This lack of hierarchical regulation was corroborated by the lack of up-regulation of mRNA concentrations of the corresponding genes. We should remark that our *in vitro* assays were performed at a single effector and substrate concentration. We therefore do not register if the affinity for substrate, product, or effector has changed, for example through the expression of a different isoenzyme for catalysis of the same reaction. These effects will therefore be included in the metabolic regulation in our analysis (38). Such an effect can be excluded for PGI, TPI, and PGK, because these enzymes have only one isoform. However, it might be relevant, for example, for HXK, where isoforms with different kinetic

³ J. Postmus and G. J. Smits, unpublished data.

properties could be expressed in a condition-specific manner, without affecting the total enzyme capacity for the corresponding reactions. For these reactions our qPCR analysis revealed significant down-regulation of one isoform and up-regulation of another. Such an exchange could for instance explain why assay temperature differently affects enzymes from cultivations at different temperatures.

Interestingly, time-resolved genome-wide expression analyses studying the response of yeast to temperature increases did reveal large changes in genome-wide expression profiles (53–55). However, these changes are mostly transient, and expression levels of most of the induced and repressed genes return to normal upon regaining homeostasis (54). We are comparing fully adapted steady state conditions. The fact that we see very little changes in glycolytic enzyme expression in these conditions does not mean that there has not been a strong hierarchically regulated adaptive response. However, for the persistently high flux at 38 °C, this mode of regulation is no longer used. Specific isoform expression, affecting flux by changing K_m rather than V_{max} , could be relevant for glucose transport. Many different hexose transporters are encoded in the yeast genome, with different catalytic properties (56). The genes are so closely related that we were not able to design distinctive qPCR primer sets for all individual genes. Therefore, in this case we did study the kinetic properties of the glucose transport system. This revealed that, besides an absence of significant changes in V_{max} , the K_m value for glucose changed from a one-component to a second component system, in which the lower affinity component was hardly contributing to the V_{max} . Taken together, these data suggest that changes in kinetic properties because of changes in glycolytic enzyme isoform expression are not responsible for the flux changes.

Because the contributions of temperature activation and hierarchical regulation were only moderate, the temperature-induced flux increase must largely be caused by changes in metabolic environment of the enzymes. Thus, the main question is how these changes can maintain the high glycolytic flux at 38 °C. At 30 °C, it is known that hexose transport has high control over glycolytic flux (57, 58). Additionally, flux control was reported for phosphofructokinase and the adenylate charge (46, 59, 60). Although control may be distributed differently at these highly increased fluxes, we evaluated these options first. First, the residual extracellular glucose concentration was 10-fold higher at 38 °C than at 30 °C. Together with the slightly altered hexose transport kinetics, this could explain an increase in hexose transport rate, if control still resides with the hexose transport step. Alternatively, at the increased temperature an increased demand for ATP might also exert control over glycolytic flux (61–64). Indeed, in a similar situation, increased ATP demand through inhibition of oxidative phosphorylation led to an increased glycolytic flux at 30 °C in *Torulopsis glabrata* (62). Both these phenomena occur simultaneously in *S. cerevisiae* at 38 °C. Interestingly, in this case *T. glabrata* needs to maintain a high flux while its end product, pyruvate, accumulates. Indeed, we measured a strongly increased pyruvate concentration (see Table 4), as well as ethanol production. The altered adenylate charge may be asserting a complex regulatory effect, as has already been observed for low temperature cultivations (52).

We propose additionally that a high flux can be maintained in the absence of a pyruvate sink by the feedback activation of PFK driven by the increased level of fructose 2,6-bisphosphate (65), and a feed-forward activation of pyruvate kinase by the increased level of fructose 1,6-bisphosphate (66, 67). This last aspect is complicated by the fact that such regulatory effects themselves may be temperature-dependent. For instance, in *Spermophilus lateralis* temperature has pronounced effects on allostery of PFK, with activating effects becoming inhibitory at lower temperatures (68).

In conclusion, our study shows that a temperature increase leads to a steep increase in glycolytic flux at temperatures above 37 °C, which is not accompanied by an increase in respiration. Regulation analysis was extended with an extra coefficient that includes the effect of temperature as physiological parameter. The observed flux increase was not because of a strong temperature dependence of enzyme activity, nor by a strong hierarchically regulated induction of enzyme activity. Therefore, the increased *in vivo* flux at 38 °C is largely regulated metabolically for all glycolytic enzymes, consistent with the observed extensive regulation of involved metabolite concentrations.

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